SCIENTIFIC REPORT

Regarding the implementation of the research project with the title: ***Xeno nucleic acids-mediated, real-time multiplexed detection of disease relevant miRNAs, with single molecule sensitivity and selectivity***, acronym RNANANODETECT, code PN-III-P4-ID-PCE-2020-0011, for all execution periods (2021-2023).

In this project we aimed to use specific xeno-nucleic acids, namely peptido-nucleic acids (PNA) conjugated with poly(Arg) arginine amino acid sequences of variable sizes, with the role of probe molecules in the multiplex detection of DNA sequences similar to target miRNAs of biological interest, using protein nanopores as transducers. The single-molecule detection method using α-hemolysin (α-HL) protein nanopore does not require labeling or amplification and is based on the complementarity between the target nucleic acid sequence and the synthesized probe nucleic acid sequence.

The single-molecule detection technique using nanopores enables the direct and real-time detection of a wide variety of molecules with low cost and low material consumption. The working principle of this approach generally follows this sequence of events: (a) by applying a potential difference across a nanopore inserted into the lipid membrane, an ionic current arises given by K^+ and Cl^- ions originating from KCl salt dissociation from the electrophysiological solution, measured along the nanopore; (b) the electric field originating from the potential difference leads the macromolecule of interest to the nanopore; (c) the transient capture of the macromolecule inside the nanopore involves changes in the electrical resistance of the nanopore, seen as fluctuations in the ionic current mediated by the nanopore.

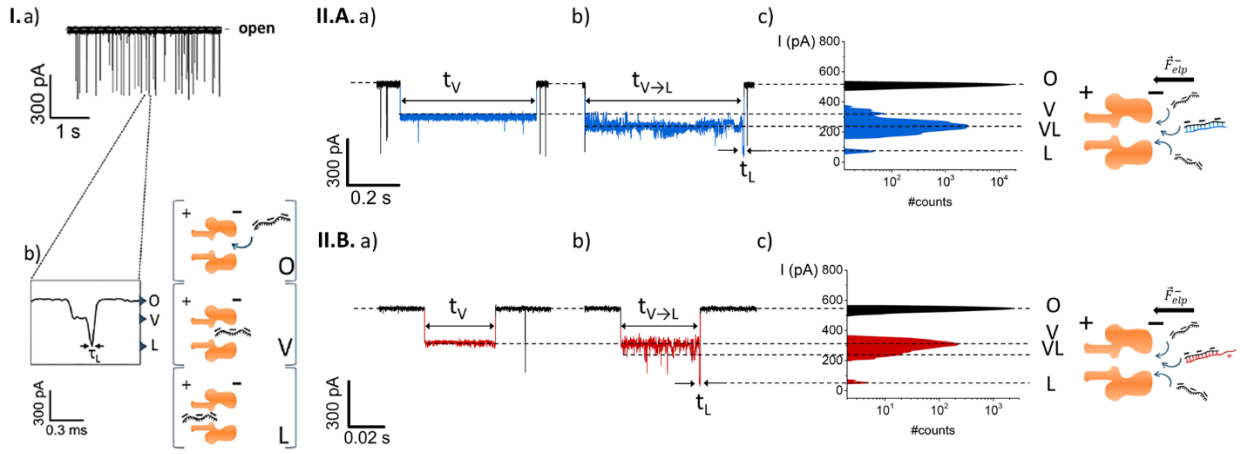

Figure 1. I. Interaction of the miRNA-like DNA target molecule, cDNA, with the α-HL protein nanopore: a) reversible fluctuations of the ion current given by cDNA target molecules, b) the specific signal of a cDNA blocking event. **II. S pecific signals of the α-HL protein nanopore interaction with the molecular duplexes A.** BPNA-cDNA and **B.** RPNA-cDNA: **a)** the complex is captured inside the vestibule and then returns to the cis region of the nanopore (t_V), **b)** the complex dissociated (t_{V-L}) into PNA molecule and cDNA molecule which will translocate further through the lumen to trans (t_L); **c)** the related amplitude histograms. *(Mereuță et al., Anal. Chem 2022, 94)*

In the first study, we analyzed the interaction between a miRNA-like DNA target molecule and two PNA probe molecules, with the help of an α-hemolysin (α-HL) protein nanopore, in order to obtain an efficient hybridization in the physiological solution. We tested the cDNA target molecule, complementary to two types of PNA probe molecules, namely BPNA containing the complementary nucleobases and RPNA, which additionally has an arginine $(R)_{10}$ polypeptide chain conjugated at the 5' terminus. This polypeptide chain gives the RPNA molecule a positive electrical charge given by the arginine amino acids at the pH used in the experimental protocol (electrophysiological KCl solution, 3M concentration, 10 mM HEPES, pH = 7.3).

Once the cDNA-PNA complex enters the vestibule of the nanopore, it either returns to the solution (**Figure 1. II, a**), or the unfolding process takes place, in which only the cDNA molecule will translocate the nanopore (**Figure 1. II, b**).Statistically analyzing the average interaction time of the complexes with the protein nanopore of α-HL we were able to investigate the stability of PNA-DNA similar to miRNA complexes and we observed that the cDNA-RPNA duplex has a lower association energy, respectively the free energy of unwinding (ΔG) is higher, due to the electric dipole structure given by the negative electric charge of the backbone of the cDNA molecule and the positive electric charge of the polyarginine sequence.

In the conclusion of the first study we can say that the method of detecting miRNA-like DNA target molecules with the help of PNA probe molecules is one with high specificity, and the presence of the polyarginine sequence attached to the PNA molecule gives us the ability to control the unfolding process of the cDNA-RPNA complex and to calculate the association energies.

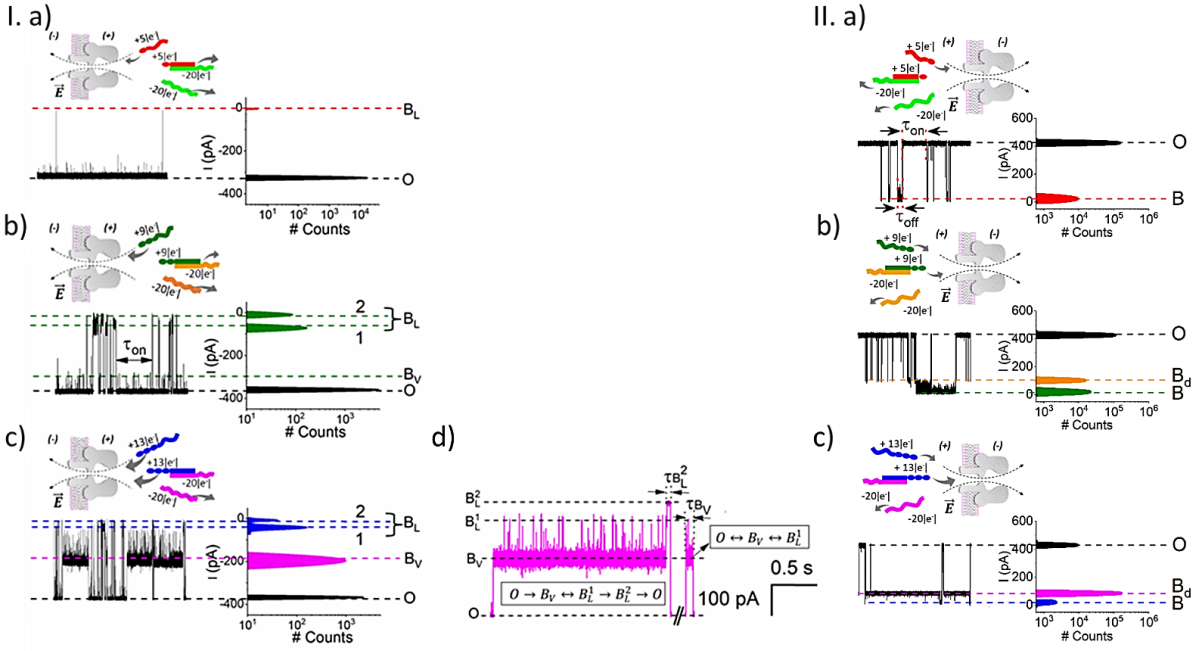

Figure 2. Detection of miRNA-like DNA target molecules following hybridization with poly(Arg)-PNA probe molecules using the α-HL protein nanopore, when the molecules of a) PA5 and cDNA(PA5), b) PA9 and cDNA(PA9), c) PA13 and cDNA(PA13) were added **I.** to cis or **II**. in trans, in concentrations of 4 μM poly(ARG)-PNA and 8 μM miRNA-like cDNA. **d)** Event types and associated sublevels that occur when PA13 and cDNA(PA13) molecules are added in cis, for 3M KCl, pH = 7. *(Mereuță et al., Anal. Chem 2022, 94)*

To select and test PNA probe molecules functionalized with polyarginine tails of different lengths in order to obtain a specific signal dependent on the length of the tail, in a second study we analyzed the interaction between a single nanopore of α-HL and probe molecules of poly (Arg)-PNAs added in concentration of 4 μM of the cis and trans side, respectively, at a concentration of 3M KCl of the recording solution and pH=7. The poly(Arg)-PNA sequences, denoted here as PA5, PA9 and PA13, are functionalized with 5, 9 and 13 Arginine amino acids at C-terminal. We observed that the hybridized complex has a specific fingerprint in the signal, denoted by B_d level in ionic current amplitude (**Figure 2.**), being a parameter used in detection process. We studied the unfolding time kinetics of the duplex and we find that the hybridization energy is higher for the duplex with a smaller number of arginines conjugated to PNA molecules, because it experiences a lower electrophoretic dehybridization force.

We tested this nanopore-based method to detect non-complementary nucleic acid sequences and obtained promising results. Starting from the probe molecule PA13 located in the trans side (**Figure 3, a**), we added the non-complementary target molecule cDNA(PA9) (**Figure 3, b**). In the recorded signal, only the B blocking level characteristic of the probe molecule PA13 was observed, a sign that the hybridization of the molecules did not occur. The cDNA(PA9) molecules are attracted in the electric field opposite to the translocation through the nanopore, so they do not appear in the ion signal. When the target molecule cDNA(PA13) was added to the system (**Figure 3, c**), a level of Bd_1 blocking specific to the PA13-cDNA(PA13) hybridized complex was observed. By adding to the solution the probe molecule PA9 complementary to the target molecule cDNA(PA9) (**Figure 3, d**), we noted a B_{d2} level, specific to PA9-cDNA(PA9) hybridized duplexes. The concentrations of poly(Arg)-PNA probe molecules and DNA target molecules were 4 and 8 μM, respectively, in 3M KCl, pH = 7.

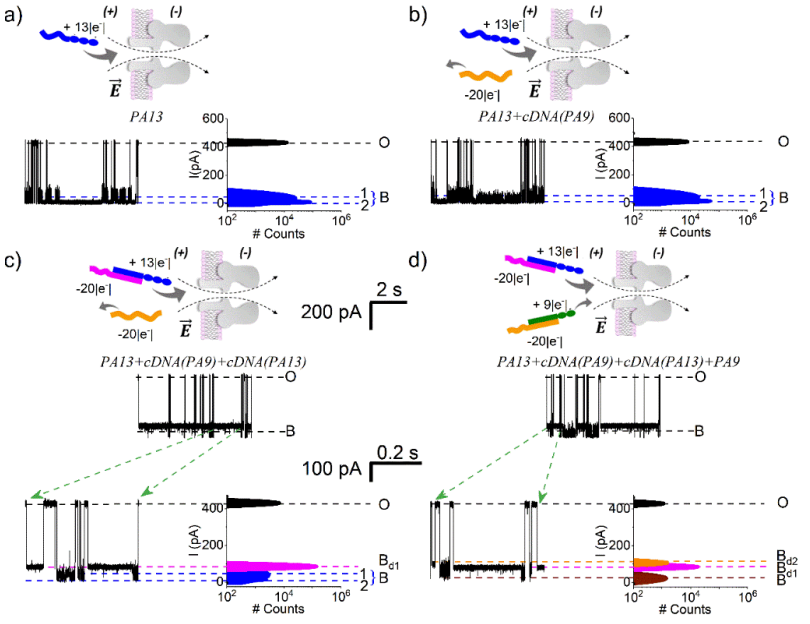

Figure 3. Multiplex detection of target DNA molecules by α-HL nanopore capture of poly(Arg)-PNA-cDNA hybridized duplexes. The signal given by the interaction of the probe molecules PA13−α-HL measured at ΔV = +140 mV (a) remains unchanged in the presence of the non-complementary target molecule cDNA(PA9) (b). Addition of complementary target molecules cDNA(PA13) caused the level of Bd1 blocking (c). Subsequent pipetting of the PA9 probe molecule, complementary to the cDNA(PA9) already present, generated hybridization of PA9-cDNA(PA9) duplexes producing the Bd2 level (d). *(Mereuță et al., Anal. Chem 2022, 94)*

In conclusion, the data obtained in the second study demonstrate that the variable-length poly(Arg) sequence in the structure of the PNA probe molecule can form the basis of a miRNA-like cDNA molecule detection system based solely on discrimination ability of the blocking

substates given by the poly(Arg)-PNA-cDNA hybridized duplex in a heterogeneous mixt. Also, two main parameters describing the association interactions of the miRNA-like PNA-cDNA duplex with the α-HL nanopore were proposed for rapid and accurate characterization of distinct miRNA-like cDNA molecules, namely the reaction constant and relative blockage.

In a third study, the proposed detection approach was also analyzed in solutions compatible with biological environments. Following the experiments within this project, it was observed that the α-HL biosensor used provides correct results even at very low analyte concentrations, which makes the detection limit of miRNA-like DNA to be of the micromolar order. In order to perform the detection in solutions compatible with biological environments, we performed experiments in which the electrophysiological solution of KCl has different concentrations: 3M, 1M and 0.5M, the latter being the closest to the concentration of K+ and Cl- ions in the body. We observed that the hybridization and stability of the complexes vary depending on the concentration of the KCl salt used, a fact also highlighted in the changes in the recorded specific ionic current (**Figure 4.**).

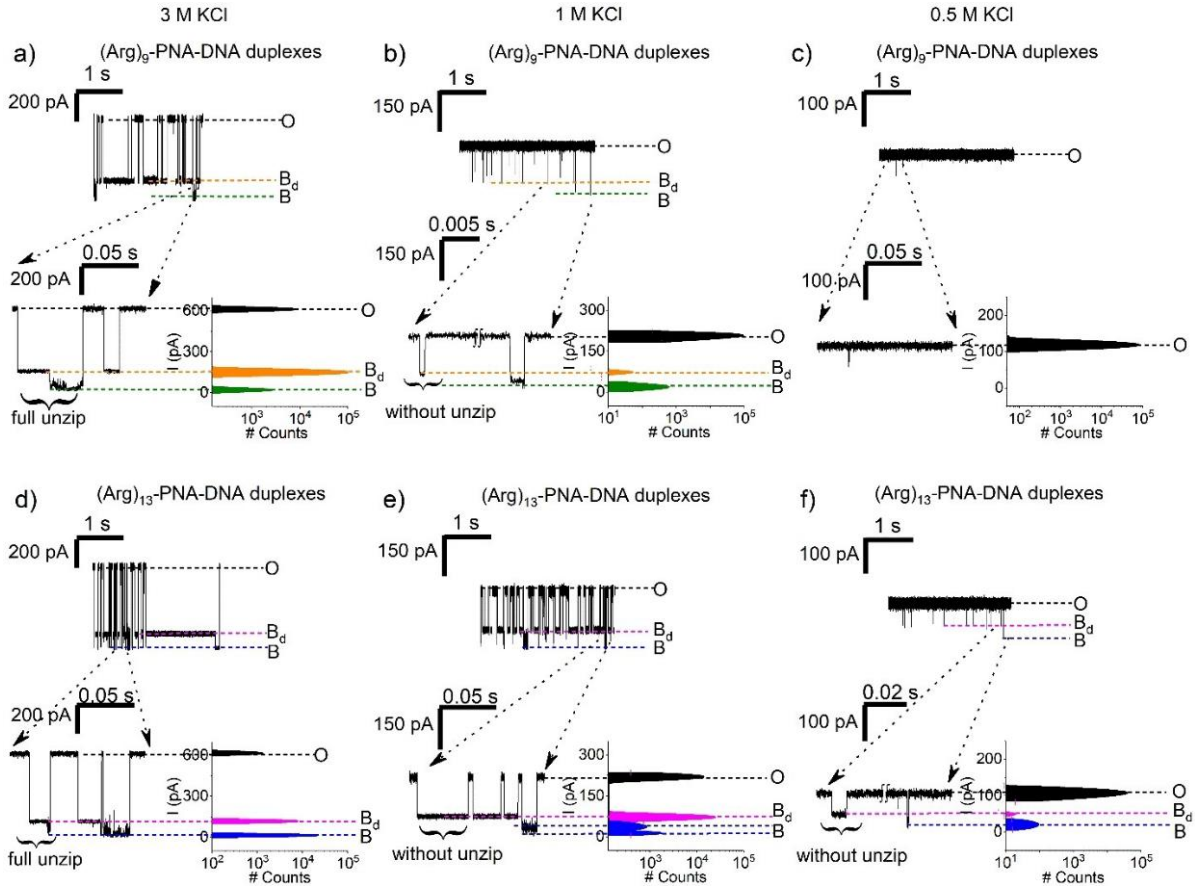

Figure 4. Detection of poly(Arg)-PNA-DNA molecular duplexes using the α-HL nanopore in media with different concentrations of KCl. Frequency and duration of blocking events elicited by PA9-cDNA(PA9) (**a-c**) and PA13-cDNA(PA13) (**d-f**) duplexes located in the trans region, at ΔV=+200mV. Representative blockage events specific to complete unfolding of the captured duplex upon entry into the α-HL lumen and duplex capture and release events without unfolding, respectively, are illustrated in the magnified images. Histograms of recorded ionic current values highlight levels of blockage. *(Asandei et al., Chem. Asian J. 2022, 17)*

We note that the value of the blocking level Bd specific to hybridized duplexes strongly depends on the concentration of KCl in the electrophysiological solution, while the value of B level specific to PNA probe molecule does not. It was observed that as the concentration of ions in the solution decreases and the electrical charges are less and less shielded, the number of hybridized duplexes decreases due to the interaction between the positive electrical charge of the poly(Arg) sequence in the PNA structure and the negative electrical charge of the sequences of DNA, preventing hybridization of the complex.

Following this stage of the study we concluded that the probe poly(Arg)-PNA molecule containing a larger number of amino acids is suitable for a wide range of concentrations of the recording solution, providing the system with high stability. Another important conclusion is that the detection strategy is also feasible in solutions similar to biological samples.

We tested in the fourth study the strategy of detecting miRNA-like single-stranded DNA molecules that is based on protein nanopores and the property of hybridization between a PNA probe sequence and a DNA target sequence, using alamethicin protein pores. They can change the number of monomers in their composition (the nanopore can have between 4 and 12 monomers), so they can have more conductive states observed in the ionic signal (**Figure 5. Ia**). When alamethicin monomers are functionalized with a PNA nucleic acid probe molecule, the conductance states are altered due to PNA interactions with ionic channels (**Figure 5. Ib, IIa**). Upon addition to the system of complementary miRNA-like DNA target molecules, the two nucleotide sequences will hybridize, reducing the mobility of alamethicin monomers, and this process will be observed in the recorded ion signal, allowing detection (**Figure 5. Ic, IIb**).

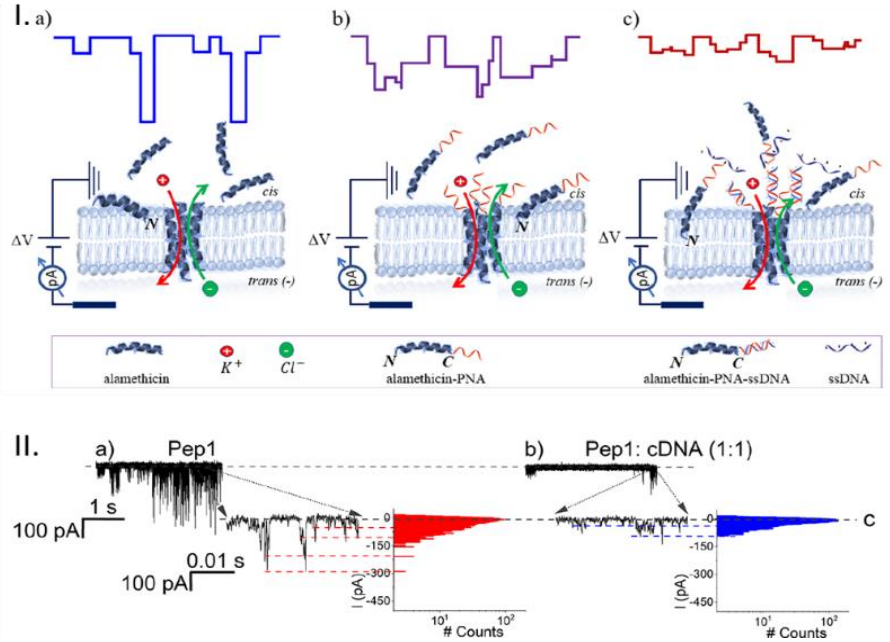

Figure 5. Detection of target DNA molecules with probe PNA molecules and alamethicin nanopores. I. Scheme of the detection principle: **a)** alamethicin nanopores have different conductive states, **b)** alamethicin monomers functionalized with PNA probe molecules produce a different signal; **c)** when complementary target DNA is added to the system, nucleic acid hybridization takes place and it can be observable in the signal. **II. a)** Original signal given by alamethicin nanopores functionalized with probe molecules called Pep1, **b)** together with cDNA target molecules *(Mereuță et al., ACS Appl. Mater. Interfaces 2023, 15)*

We were thus able to fulfill the objectives we proposed and demonstrate the possibility of multiplex analysis of the profiles of different DNA molecules similar to miRNA, in electrolytic solutions and solutions similar to biological samples using the detection method based on protein nanopores and the hybridization process between DNA target molecules and PNA probe molecules functionalized with polyarginine sequences of various lengths. We were also able to obtain with this method a DNA similar to miRNA detection limit in the micromolar order. The presented results demonstrate the usefulness and feasibility of methods for detecting molecules of biological interest using protein nanopores and underline the importance of bionanotechnologies in the development of modern diagnostic and treatment methods.

Summary of progress (achieved deliverables, result indicators, dissemination of results, and justification of differences, if applicable).

Verifiable results of activity	Estimated	Realized
Articles	2	9
Participation in conferences	2	2
Web page	1	1
Work protocol	1	4

Results and manner of dissemination of results

A total of 9 articles published in journals with impact factor, from which 7 located in the red zone (Q1) and 1 located in the yellow zone (Q2).

1. Mereuta, Loredana; Asandei, Alina; Schiopu, Irina; Park, Jonggwan; Park, Yoonkyung; Luchian, Tudor *Synthetic Receptor Based on a Peptide Antibiotic-Functionalized Chimera for Hybridization-Based Polynucleotide Detection*, ACS APPLIED MATERIALS & INTERFACES 2023, 15 (27), 33159-33168, DOI:10.1021/acsami.3c06086, **Q1**
2. Mereuta, Loredana; Asandei, Alina; Andricioaei, Ioan; Park, Jonggwan; Park, Yoonkyung; Luchian, Tudor, *Considerable slowdown of short DNA fragment translocation across a protein nanopore using pH-induced generation of enthalpic traps inside the permeation pathway*, NANOSCALE 2023, 15(36), 14754-14763. DOI: 10.1039/d3nr03344a. **Q1**
3. Loredana Mereuta, Alina Asandei, Isabela Dragomir, Jonggwan Park, Yoonkyung Park, and Tudor Luchian. *A Nanopore Sensor for Multiplexed Detection of Short Polynucleotides Based on Length-Variable, Poly-Arginine-Conjugated Peptide Nucleic Acids*. Analytical Chemistry 2022, 94 (24), 8774-8782, DOI: 10.1021/acs.analchem.2c01587, **Q1**
4. Alina Asandei, Loredana Mereuta, Ioana C. Bucataru, Yoonkyung Park, Tudor Luchian. *A Single-Molecule Insight into the Ionic Strength-dependent, Cationic Peptide Nucleic Acids-Oligonucleotides Interactions.* Chemistry An Asian Journal 2022, e202200261, DOI: 10.1002/asia.202200261, **Q2**

5. Ioana C. Bucataru, Isabela Dragomir, Alina Asandei, Ana-Maria Pantazica, Alina Ghionescu, Norica Branza-Nichita, Yoonkyung Park, Tudor Luchian. *Probing the Hepatitis B virus e-antigen with a nanopore sensor based on collisional events analysis.* Biosensors 2022, 12 (8), 596. **Q1**
6. Schiopu Irina, Asandei Alina, Mereuta Loredana, Dragomir Isabela, Bucataru Ioana Cezara, Luchian Tudor. *Single-molecule detection and manipulation with biological nanopores*. Studia Universitatis Babes-Bolyai, Chemia . 2021, 66 161-174, **Q4**
7. Alina Asandei, Loredana Mereuta, Irina Schiopu, Yoonkyung Park, Tudor Luchian. *Teaching an old dog new tricks: A lipid membrane-based electric immunosensor for real-time probing of the spike S-1 protein subunit from SARS-CoV-2*. Proteomics, 2021, e2100047, **Q1**
8. Tudor Luchian, Loredana Mereuta, Yoonkyung Park, Alina Asandei, Irina Schiopu. *Single-molecule, hybridization-based strategies for short nucleic acids detection and recognition with nanopores*. Proteomics, 2021, e2100046. **Q1**
9. Isabela S Dragomir, Alina Asandei, Irina Schiopu, Ioana C Bucataru, Loredana Mereuta, Tudor Luchian. *The Nanopore-Tweezing-Based, Targeted Detection of Nucleobases on Short Functionalized Peptide Nucleic Acid Sequences*. Polymers, 2021, 13, 1210, **Q1**

The results obtained during this stage were presented at **1 international conference** and **1 national conference**:

1. Sixth Edition of International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences, "IC-ANMBES 2022", June 8-10, 2022, Brasov, Romania

- *Detection of nucleobases on short functionalized peptide-nucleic acid sequences using nanopore-tweezing method*, Isabela S. Dragomir, Alina Asandei, Irina Schiopu, Ioana C. Bucataru, Loredana Mereuta, Tudor Luchian
- *Protein nanopore-based method for sequence specific detection of single-stranded DNA using gold nanoparticles and peptide nucleic acids*, Ioana Cezara Bucataru, Loredana Mereuta, Alina Asandei, Isabela Dragomir, Tudor Luchian

2. A XVII-a Conferinţă Naţională de Biofizică, CNB 2022, 23-25 Septembrie 2022, Târgu Mureș, România

- *A tug-of-war between electric forces: The nanopore-tweezing method applied in molecular sensing*, Isabela S. Dragomir, Alina Asandei, Irina Schiopu, Ioana C. Bucataru, Loredana Mereuta, Tudor Luchian

Date — Projec Director,

15.11.2023 — Prof. Univ. Dr. Tudor LUCHIAN